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The Effects of Stress and Alcohol on HIV-1 Latency

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LOYOLA UNIVERSITY CHICAGO

THE EFFECTS OF STRESS AND
ALCOHOL ON HIV LATENCY

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
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MASTERS OF SCIENCE

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BY
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LIST OF ABBREVIATIONS

AP-1	Activator protein 1
APOBEC3G	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G
Aza-CdR	5-Aza-2'-deoxycytidine
CCR5	C-C chemokine receptor type 5
CD	Cluster of differentiation
CpG	Cytosine-phosphate-guanine
CXCR4	C-X-C chemokine receptor type 4
Dex	Dexamethasone
DNMT	DNA methyl transferases
Env	HIV-1 envelope
EtOH	Ethanol
FACS	Fluorescence activated cell sorting
GC	Glucocorticoid
GFP	Green fluorescent protein
gp	glycoprotein
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HAART	highly active antiretroviral therapy
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HIV	Human immunodeficiency virus
HPA	Hypothalamic-pituitary-adrenal
I κ B	Inhibitor of κ B
IL-2	Interleukin 2

JAK	Janus kinase
LTR	Long terminal repeat
NK	Natural killer cells
PCR	Polymerase chain reaction
PBMC	Peripheral blood mononuclear cell
PIC	Pre-integration complex
NFAT	Nuclear factor of activated T-cells
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NT	No treatment
ROS	Reactive oxygen species
RT	Reverse transcription
SAHA	Suberoylanilide hydroxamic acid (Vorinostat)
SAM	S-adenosyl methionine
Ser-	Serine
STAT	Signal transducer and activator of transcription
TNF α	Tumor necrosis factor alpha
TAR	Trans-activation response element
TPA	12-O-Tetradecanoylphorbol-13-acetate
TSA	Trichostatin A
VPA	Valproic acid
VSVg	Vesicular stomatitis virus glycoprotein G

CHAPTER ONE

OVERVIEW AND HYPOTHESES

A major barrier to HIV eradication is the persistence of latent viral reservoirs that exist despite antiretroviral therapy. Latent reservoirs are primarily made up of memory CD4⁺ T cells, which become infected while activated and survive long enough to revert to a quiescent state that is non-permissive for viral gene expression (Siliciano et al., 2011). Current HIV research is focused on eliminating this pool of latently infected cells by the “shock and kill” method, which involves purging the virus out of latency using a variety of compounds that force viral gene expression (Jordan et al., 2003; Colin et al., 2009). Cells producing virus will be targeted by host immune responses, while antiretroviral drugs prevent new rounds of infection. However, this method has proven difficult since these drugs are unable to reactivate each latent provirus, leading some to suggest that HIV latency and reactivation is stochastic (Ho et al., 2013; Weinberger et al., 2013).

Stress and alcohol consumption are prevalent among HIV-infected individuals, having deleterious effects on the immune system (Glasser et al., 2005; Justice et al., 2013). Glucocorticoids and ethanol mediate their effects by altering cell signaling pathways, the activity of transcription factors (NF- κ B, AP-1), and inducing epigenetic modifications that alter the expression of immune response genes and consequently,

immune cell function (Nelson et al., 2003; Webster et al., 2002; Krukowski et al., 2011; Curtis et al., 2013; Arror et al., 2004; Thannickal et al., 2000). These glucocorticoid and ethanol-induced modifications likely have profound effects on HIV latency since transcription factor availability and epigenetic mechanisms play a role in the establishment, maintenance, and reactivation of latently infected cells.

In order to develop effective therapeutics, a comprehensive understanding of latency and factors driving the formation of the latent reservoir is needed. As stress and alcohol are common comorbidities associated with HIV infection, the goal of this research was to determine how stress and alcohol could affect HIV-latency. Specifically, we hypothesized that the ability of latent proviruses to be reactivated by “shock and kill” approaches would be altered by glucocorticoid and ethanol treatments, and prolonged ethanol exposure would affect the size of the latent reservoir. To test our hypothesis, we examined two aspects of latency. In **aim 1** we assessed whether viral reactivation was altered by dexamethasone and ethanol treatments. We then addressed how dexamethasone and ethanol exposure could affect the establishment of latent reservoirs in **aim 2**.

CHAPTER TWO

INTRODUCTION

Human immunodeficiency virus-1 (HIV-1) is the causative agent of Acquired Immunodeficiency Syndrome (AIDS), which caused more than 1.5 million deaths worldwide in 2013 alone (World Health Organization, 2014). According to the World Health Organization, an estimated 35 million people are currently living with HIV (World Health Organization, 2014). Because this virus targets immune cells, namely CD4⁺ T cells, the depletion of the cells overtime leads to immunosuppression and gives rise to opportunistic infections, to which patients eventually succumb. The introduction of highly active antiretroviral therapy (HAART) has significantly reduced the morbidity and mortality rates, as it slows the loss of these immune cells and progression to AIDS (Van Lint et al., 2013). Unfortunately, HAART is unable to completely eradicate HIV infection, as any interruption in therapy results in a rapid viral rebound. This is because infected patients harbor transcriptionally silent but replication-competent viral reservoirs that exists even in the continued presence of antiretroviral drugs (Van Lint et al., 2013).

HIV INFECTION CYCLE

Inside each HIV viral particle are two identical positive sense RNAs associated with multiple proteins. Group antigen (gag) proteins make up the structural elements of

the virus and include the nucleoproteins that surround the RNA. A number of enzymes are present in the core, including reverse transcriptase, ribonuclease H, protease, and integrase. Surrounding the core is a lipid envelope comprised of glycoproteins gp120 and gp41. These structural proteins, enzymes, and envelope glycoproteins are encoded in three genes in the HIV genome, *gag*, *pol*, and *env*, respectively.

The HIV genome also includes accessory genes *vif*, *vpu*, *vpr*, and *nef* and regulatory genes *tat* and *rev*. The proteins encoded by these genes carry out many important functions for the HIV infection cycle. Briefly, Vif plays a role in viral replication and promotes the degradation of APOBEC3G, a restriction factor that hypermutates viral cDNA, preventing efficient infectivity (Trono, 1995; Rose et al., 2004). Vpu stimulates virion release and CD4 degradation. Vpr aids in the infection of non-dividing cells, such as terminally differentiated macrophages, and can induce cell cycle arrest (Trono, 1995). Nef enhances HIV-1 infectivity and triggers CD4 down-regulation (Trono, 1995). Rev regulates transport of nascent viral mRNAs into the cytoplasm for translation (Uberla, 2010). Tat acts as a potent transcriptional activator. Upon translation, Tat comes back into the nucleus, binds the trans-activating response element (TAR), and recruits cellular transcription machinery to initiate effective transcription of the entire viral genome from the 5'LTR (Romani et al., 2010).

Viral entry occurs at mucosal surfaces, primarily through sexual contact. Gp120 binds CD4 receptors, causing a conformational change that allows binding to a coreceptor, either CCR5 or CXCR4 (Wilén et al., 2012; Doms et al., 2000). Following coreceptor binding, gp41 becomes exposed and penetrates the target cell membrane,

allowing the two membranes to come together and ultimately fuse (Doms et al., 2000; Engelman et al., 2012).

After HIV fuses to the target cell, the viral core gets released into the host cell cytoplasm and reverse transcription is initiated. tRNAs hybridized to the viral RNAs prime cDNA synthesis and reverse transcriptase converts the RNAs to double stranded DNA (Hu et al., 2012). Ribonuclease H (RNase H) degrades RNA after it has served as a template for reverse transcription (Hu et al., 2012). The karyophilic pre-integration complex (PIC) enters the nucleus, and integrase embeds the provirus into the host cell chromosome, preferentially in areas of active transcription near the nuclear periphery (Craigie et al., 2012; Albanese et al., 2008). At this point, the virus can be transcribed. RNA polymerase II and associated transcription factors bind the HIV-LTR and drive transcription of gag, pol and env. Spliced mRNAs escape into the cytoplasm, and unspliced or incompletely spliced viral RNAs get exported from the nucleus with the help of Rev (Sundquist et al., 2012).

The viral glycoproteins are synthesized from an env/vpu mRNA transcript that is translated on the rough ER. The glycoprotein precursor (gp160) becomes glycosylated, oligomerizes and traffics to the Golgi where it is further processed into gp120 and gp41 (Checkley et al. 2012). These glycoproteins traffic via the secretory pathway and are deposited on the plasma membrane. Gag-pol mRNA translates into multiple gag proteins and a gag-pol polyprotein generated by translational frameshifting (Sundquist et al., 2012). This gag-pol polyprotein houses the viral protease, reverse transcriptase and integrase.

Translated products, including two copies of viral RNA, cellular tRNA, and the viral structural and enzymatic proteins (gag proteins and gag-pol polyprotein) assemble at the plasma membrane (Sundquist et al., 2012). The envelope is acquired from the host cell membrane as the virus buds. Maturation is a largely extracellular event driven by the viral protease. Immature virions that bud out from a host cell are not infectious. PR cleaves specific sites within Gag, so that Gag constituent proteins can condense and rearrange forming the conical core of a mature virus (Sundquist et al., 2012). Mature viruses can go on to infect other cells, and the cycle continues.

HAART is comprised of a cocktail of drugs such as reverse transcription inhibitors, protease inhibitors, fusion inhibitors and integration inhibitors, which are designed to target different phases of the HIV life cycle (Engleman et al., 2012). The concomitant administration of these drugs is intended to prevent the emergence of antiretroviral-drug resistant viruses. While HAART has successfully slowed progression to AIDS, there are many side-effects to long term antiretroviral (Van Lint et al., 2013). Unfortunately, cessation of therapy is not an option, as any interruption in HAART is followed by rapid viral rebound. This is due to the existence of latent reservoirs.

HIV LATENCY

Background

After entry and integration, some viruses get transcribed and enter the productive infection cycle described above. Alternatively, some proviruses enter a latent state. The latent reservoir is primarily comprised of memory CD4⁺ T cells (Siliciano et al., 2011; Donahue et al., 2013). HIV has high tropism for activated CD4⁺ T cells. Once infected,

many of these cells undergo cell death due to cytotoxic effects of the virus or recognition by host immune responses. A subset of infected cells survive and enter a quiescent or memory state, forming long-lived memory T cells (Siliciano et al. 2011). This latent reservoir is intrinsically stable and the progressive loss of these cells is very slow (Siliciano et al., 2011; Van Lint et al., 2013). It has been estimated that it would take over 70 years to eradicate this pool of latently infected cells from a patient on HAART (Siliciano et al., 2011). As a result, patients have to remain on antiretroviral drugs for the remainder of their lives.

Latency is established reproducibly during HIV infection. The number of latently infected cells in a HIV-infected individual is low, ranging between 1-10 million cells per individual (Jordan et al., 2003). Current HIV research is focused on eliminating this pool of latently infected cells, as they represent a major barrier to viral eradication. One proposed eradication strategy involves purging the virus out of latency using a variety of compounds that force viral gene expression. The idea here is that reactivated cells will begin to produce virus and will be eliminated by host immune responses and/or HIV-mediated cell death, while new rounds of infection will be prevented by antiretroviral drugs (Jordan et al., 2003; Colin et al., 2009; Van Lint et al., 2013). The numerous mechanisms maintaining the latent state (such as transcriptional interference, absence of host transcription factors, numerous repressive epigenetic modifications, etc.) make complete eradication by this ‘shock and kill’ approach unlikely (Siliciano et al., 2011; Colin et al., 2009). In theory, a single latently infected cell can cause viral rebound, meaning that complete eradication requires drugs that will induce viral expression in *all* latently infected cells. The more realistic goal of viral purging would be to reduce the size

of the latent reservoir to a level where the immune system can control the virus, at least long enough to allow interruptions in antiretroviral treatments (Colin et al., 2009).

Mechanisms of latency and proviral reactivation

Chromatin remodeling mechanisms are a major determinant in transcription of the HIV genome. Two nucleosomes, nuc-0 and nuc-1, are important regulators of HIV transcription. They are positioned near the HIV-LTR and overlap with important transcription factor binding sites (Siliciano et al., 2011). Remodeling of nuc-1, which is positioned just downstream of the transcription start site, is associated with viral reactivation (Shirakawa et al., 2013; Siliciano et al., 2011; Van Lint et al., 2013). Importantly, treatment with HDAC inhibitors alone allows for this remodeling of nuc-1 and subsequent viral reactivation (Shirakawa et al., 2013; Siliciano et al., 2011).

Histone deacetylases (HDACs) repress transcription by deacetylating histone proteins near the viral promoter. These HDACs get recruited to the HIV-LTR by various transcription factors, such as Ying-Yang 1 (YY1), late SV40 factor (LSF), COUP-TF interacting protein (CTIP2), c-promoter-binding factor-1 (CBF-1), thyroid hormone receptor, NF- κ B p50 homodimer, and c-myc and Sp1 (Shirakawa et al., 2013; Siliciano et al., 2011). Histone deacetylase inhibitors activate transcription by inhibiting the activities of HDACs. Inhibition of class I HDACs by HDAC inhibitors such as Trichostatin A (TSA), Vorinostat (SAHA), Valproic acid (VPA) and a number of other drugs have been studied in regards to HIV latency. SAHA and VPA had moderate success in some clinical trials, but failed to induce viral reactivation in subsequent studies (Shirakawa et al., 2013; Spina et al., 2013; Siliciano et al., 2011).

DNA methylation of CpG islands that flank the transcription start site also contribute the proviral latency. Consistent with this, chromatin associated with H3K9me2 and H3K27me3 histone modification marks found near the nuclear periphery in lamina-associated domains (LADs) (Kind et al., 2010). Genes in these LADs have very low expression (Kind et al., 2010). The mechanism of methylation involves recruitment of the methyl binding protein MBD2 and the nucleosome remodeling and histone deacetylation (NuRD) complex (Shirakawa et al., 2013; Spina et al., 2013; Siliciano et al., 2011). Aza-CdR, a DNA methylation inhibitor, acts synergistically with NF- κ B activators to induce viral reactivation (Blazkova et al., 2009; Siliciano et al., 2011).

A number of host transcription factors are essential for transcription from the HIV-LTR. NFAT, NF- κ B and AP-1 are sequestered in the cytoplasm of resting T cells, thus coupling proviral transcription with T cell activation (Siliciano et al., 2011; Van Lint et al., 2013; Coffin et al., 1997). Another transcription factor found to be important in viral reactivation is Sp1. Sp1 remains inactive in quiescent cells, but upon entry into the cell division cycle, it becomes activated and initiates proviral transcription (Bosque et al., 2009). Many drugs used to purge latent reservoirs target these transcription factors to initiate viral reactivation, such as TNF α and TPA, which activate NF- κ B and PKC, respectively.

Models of HIV latency

Latently infected cells *in vivo* are present in low numbers and are phenotypically indistinguishable from uninfected cells, making studying HIV latency difficult (Spina et al., 2013). A number of *in vitro* and *ex vivo* models of HIV latency have emerged in the

past decade, that have been useful for studying how latency gets established, mechanisms maintaining the latent state, and identifying latency reversing agents for viral reactivation. The J-Lat cell model developed by Verdin and colleagues has been widely used to study mechanisms of HIV latency and the efficacy of potential latency reversing agents. J-Lat cells were derived by infecting Jurkat T cells with an HIV-based retroviral vector containing wild-type *tat* and a GFP reporter (Jordan et al., 2003). Four days after infection, GFP-negative cells were selected by FACS. To separate the latently infected from the uninfected cells, cells were treated with TPA or TNF α to activate HIV expression, and the GFP-positive cells were isolated by FACS. This population of cells represents latently infected cells that only express viral proteins after activation (Jordan et al., 2003). Individual cells were cloned and have been studied particularly for mechanisms of viral reactivation, which can be easily monitored by flow cytometry.

While latently infected T cell lines have contributed substantially to the field, there are disparities across model systems, as a single cell model is unable to fully recapitulate the biological characteristics of latently infected cells in HIV-infected patients (Spina et al., 2013). Primary cell models more accurately reflect the properties of latently infected cells *in vivo* and are therefore important for studying HIV latency. The Bosque and Planelles model involves the establishment of latently infected effector and central memory T cells (Bosque et al., 2009). In this model, naïve CD4⁺ T cells isolated from PBMCs are activated with anti-CD3 and anti-CD28 and primed toward differentiation into Th1, Th2 and non-polarized subsets (Bosque et al., 2009). Differentiated cells are infected, and latency is established by day 7 after infection (Bosque et al., 2009). Another primary cell model developed by the Karn and colleagues

obtained a population of latently infected CD4⁺ T cells by infecting activated CD4s with a lentiviral vector expressing a GFP reporter, isolating the GFP-positive cells by FACS, and cultivating infected cells on feeder cells to allow transcriptional silencing (Tyagi et al., 2010). The result is a population of latently infected cells with a quiescent phenotype that can be maintained in culture for months (Tyagi et al., 2010).

Stochasticity of latent proviruses

It has recently been proposed that the reactivation of HIV from latent reservoirs is stochastic (Ho et al., 2013). The stochastic model suggests that viral entrance into latency occurs by chance, and viral reactivation is therefore probabilistic. This idea comes from multiple findings that not all latent viruses become reactivated upon cellular activation. The Siliciano group analyzed various factors that could potentially explain why these intact proviruses were not being expressed in activated cells, while identical proviruses were reactivated (Weinberger et al., 2013). They found that while most non-induced viruses were defective (mediated by APOBEC3G-induced hypermutation), some proviruses remained silent despite cellular activation. These proviruses were not integrated into heterochromatin, there was little CpG methylation at the viral promoter, and transcriptional interference did not account for the proviral silencing. As a result of these findings, they concluded that the reactivation of latent proviruses is stochastic, even after maximum activation (Ho et al., 2013).

STRESS AND IMMUNE FUNCTION

Numerous studies demonstrate that stress influences immune function and can contribute to disease progression (Capitanio et al., 1998; Glasser et al., 2005; Krukowski

et al., 2011.). As stressors are perceived in the brain, the hypothalamic-pituitary-adrenal (HPA) axis is stimulated, resulting in the release of adrenocorticotrophic hormones by the pituitary gland and the production of glucocorticoid hormones. Release of glucocorticoids into the circulation can result in modulation of the immune system, as glucocorticoid receptors (GR) are expressed by most immune cells (Glassar et al., 2005). In these cells, the glucocorticoid hormones bind their cognate receptor, activating it. Ligand-activated GR rapidly translocates into the nucleus where it dimerizes with another activated GR. The homodimer binds glucocorticoid response elements (GREs) in the promoter of GC-sensitive genes and activates transcription (Shacke et al., 2004). The HIV promoter contains GRE-like sequences, so it is possible that glucocorticoid exposure could have a positive effect on viral transcription (Coffin et al., 1997; Kino et al., 2000). Additionally, the viral protein Vpr has been shown to enhance the effect of glucocorticoids by interacting directly with the GR (Kino et al., 2000; Kino et al., 1999).

Glucocorticoids can also repress the expression of certain genes, particularly those involved in immune responses (Nelson et al., 2003). In the nucleus, the GR can form a heterodimer with transcription factors, such as NF- κ B and AP-1, preventing transactivation of their target genes (Webster et al., 2002). The transrepressive effects of activated GR also occur in the cytoplasm, where the GR indirectly prevents the translocation of transcription factors into the nucleus. For example, ligand-activated GR induces synthesis of I κ B, which sequesters NF- κ B in the cytoplasm (Auphan et al., 1995). Additionally, glucocorticoids can mediate their anti-inflammatory effects by inhibiting signaling cascades (Nelson et al., 2003). A number of cytokines, such as IL-2, elicit their effects via the Jak-STAT signaling pathway, and inhibition of this signaling

cascade by glucocorticoid treatment has suppressive effects on immune cell function (Bianchi et al., 2000). The primary way glucocorticoids dysregulate immune function is by inducing epigenetic modifications that alter the activation status of immune response genes. Specifically, glucocorticoids actively recruit histone deacetylase complexes and inhibit histone acetyltransferase activity, resulting in histone deacetylation and reduced gene expression. This has been shown in natural killer cells, where treatment with dexamethasone, a synthetic glucocorticoid, results in decreased natural killer cell activity (Krukowski et al., 2011).

Stress has previously been shown to accelerate HIV disease progression. One study showed that socially stressed rhesus macaques infected with simian immunodeficiency virus (SIV) had higher concentrations of viral RNA in the plasma and died more quickly compared to infected rhesus macaques in stable social conditions (Glasser et al., 2005; Capitanio et al., 1998). Another study determined that HIV-infected men who had experienced stressful life events or lacked an adequate support system more quickly developed AIDS (Glasser et al., 2005; Leserman et al., 1999). Stress has been found to increase reactivation and enhance lytic viral replication in a number of other viruses including herpes simplex virus (HSV), Epstein-Barr virus (EBV), and cytomegalovirus (CMV), so it is possible that stress may have similar effects on the reactivation of HIV-latently infected cells (Glasser et al., 2005).

It is unclear if stress will affect the establishment of latency and/or viral reactivation. The viral promoter contains GRE-like sequences, so transcription from the LTR may be activated by glucocorticoid treatment (Coffin et al., 1997). However, other transcription factors present at the viral promoter may influence hormone responsiveness

(Coffin et al., 1997). GR can directly or indirectly alter the activities of NF- κ B and AP-1. Since viral transcription heavily relies on the activity of these host factors, glucocorticoid exposure may prevent viral reactivation and enhance the establishment of latency. Along with this, glucocorticoid treatment could induce epigenetic modifications, like deacetylation of histones near the viral promoter, to augment this latent state. Increased viral replication has been seen in more clinical models of stress and HIV and in studies on other viruses as well. Therefore, the possibility that glucocorticoids may enhance viral reactivation and reduce entrance into latency should not be eliminated.

ALCOHOL AND IMMUNE FUNCTION

Alcohol use is prevalent among HIV-infected individuals, and it is often associated with increased viral progression to AIDS (Justice et al., 2013; Molina et al., 2015). Alcohol consumption can have a number of effects in different cell types, including chromatin remodeling and the generation of reactive oxygen species. These alcohol-induced changes may potentially effect on the establishment, maintenance and reactivation of latently infected cells.

Alcohol consumption induces a number of epigenetic modifications including histone acetylation, methylation and phosphorylation. The metabolism of alcohol leads to the production of acetate, which gets converted to acetyl CoA, a cofactor for histone acetyl transferases (HATs) (Samir, 2013). The resulting increase in HAT activity causes histone acetylation of histone H3 lysine 9 in a variety of tissues (Park et al., 2003; Kim and Shukla, 2006). DNA methylation can also be affected by alcohol consumption. Chronic ethanol exposure reduces S-adenosyl methionine (SAM) activity and inhibits

DNA methyl transferases (DNMTs), causing a global reduction in DNA methylation (Samir, 2013). In hepatocytes, ethanol treatment causes a reduction in histone H3 lysine 9 methylation and an increase in histone H3 lysine 4 methylation. Alcohol and its metabolite acetaldehyde have been shown to phosphorylate histone H3 at ser-10 and ser-28 (Park et al., 2003) and this phosphorylation is mediated by p38 MAP kinase (Shukla et al. 2008). All of these epigenetic marks are associated with transcriptional activation, which causes an increased production of pro-inflammatory cytokines and promotes exaggerated inflammatory responses (Curtis et al., 2013). This unchecked inflammation can lead to multi-organ failure and death (Choudry et al., 2008). Recently, one group demonstrated that while binge drinking causes the down-regulation of various classes of HDACs, there is actually a binge-ethanol mediated increase in hepatic HDAC3 expression, contributing to liver steatosis and injury in ethanol-treated mice (Kirpich et al., 2013). Notably, treatment with TSA attenuated HDAC3 expression and liver steatosis (Kirpich et al., 2013).

Ethanol metabolism also produces reactive oxygen species (ROS) in numerous cell types. Many cell signaling pathways are regulated by oxidative stress. ROS signaling activates map kinase pathways leading to AP-1 and NF- κ B activation and stimulates the release of intracellular Ca^{2+} , which results in nuclear translocation of NFAT (Arrar et al., 2004; Thannickal et al., 2000). The effects of ethanol-induced generation of ROS on NF- κ B activation have been studied in regards to HIV latency. Ethanol treatment resulted in TNF α -mediated activation of NF- κ B and enhanced HIV-1-LTR activity. ROS caused indirect activation of NF- κ B by enhancing the degradation of I κ B α , thus allowing NF- κ B nuclear translocation (Dong et al., 2000).

Enhanced HIV replication due to alcohol treatment has been demonstrated in a number of studies. Acute alcohol consumption leads to dysregulation of immune function and increases susceptibility to bacterial and viral infections (Molina et al., 2015). Alcohol has been shown to significantly increase HIV infection of cord blood monocyte derived-macrophages by inhibiting the expression of HIV-1 restriction factors such as APOBEC3G (Mastrogiannis et al., 2014). A study on SIV-infected rhesus macaques demonstrated increased viral load in alcohol-dependent animals compared to control groups (Kumar et al., 2005). Other studies have noted significant increases viral replication in individuals exposed to alcohol prior to HIV infection (Molina et al., 2015).

The specific effects of ethanol on HIV-latently infected CD4⁺ T cells remains to be determined. The epigenetic modifications induced by ethanol and the effects of alcohol exposure in general are multifactorial and tissue specific. Additionally, ethanol elicits different outcomes depending on the duration of the exposure and amount consumed (Goral et al., 2008). Increased levels in HIV replication may decrease the establishment of the latent reservoir, as more viruses are being propagated and more cells are becoming productively infected. Along with this, emerging data suggests that ethanol treatment increases transcription from the HIV-LTR and therefore should enhance viral reactivation (Dong et al., 2000).

SIGNIFICANCE

The existence of latent reservoirs in HIV-infected individuals represents a major barrier to viral eradication, and current research is focused on eliminating these latently infected cells. Viral purging with latency reversing agents has seen some success *in vitro*, but clinical trials have yielded mixed results. Even though it is clear that multiple

molecular mechanisms are involved in maintaining the latent state, emerging evidence suggests that proviral latency is a stochastic event.

Glucocorticoids and ethanol exposure could have profound effects on HIV latency. Both stimuli alter cell signaling pathways involved in proviral reactivation, induce chromatin remodeling near the viral promoter, and potentiate or inhibit the activities of host transcription factors necessary for transcription of the viral genome. These glucocorticoid and ethanol-induced modifications might explain the apparent stochasticity attributed to the establishment of latent reservoirs and proviral reactivation. Given the importance of HIV latency to viral eradication and that stress and alcohol are often concomitant with HIV infection, the potential impact of these comorbidities on various aspects of latency warrants further investigation. Insight into the mechanisms of how stress and alcohol affect the latent reservoir may be important in the development of effective therapeutics.

CHAPTER THREE

METHODS

Cell culture and drug treatments

J-Lat, Jurkat, and CD4⁺ T cells were cultured in RPMI-1640 medium (Cellgro) supplemented with 10% fetal bovine serum (FBS), 1,000 U/ml penicillin, 1,000 U/ml streptomycin and 10 ug/ml ciprofloxacin hydrochloride. 100 U/ml of recombinant human IL-2 (R&D systems) was added to primary cell cultures. Dexamethasone (Sigma-Aldrich) was used at final concentrations ranging from 10⁻⁵ M to 10⁻¹⁰ M and ethanol was used at final concentrations ranging from 17 mM to 65 mM.

Isolation and activation of CD4⁺ T cells

CD4⁺ T cells were isolated from peripheral blood mononuclear cells obtained from unidentified donors. Briefly, PBMCs were isolated from whole blood by density gradient separation using Lymphocyte Separation Medium (Corning). CD4⁺ T cells were separated from PBMCs by negative isolation using a CD4⁺ T cell isolation kit and LD or autoMACS columns (Miltenyi). CD4⁺ T cells were resuspended in RPMI-1640 with IL-2 and placed in 24 well plates pre-coated with 2.5 ug/ml of anti-CD3 (OKT3, eBiosciences) and 2.5 ug/ml anti-CD28 (CD28.2, BD Biosciences). Cells were stimulated for 3 days.

Virus production and infection

293T cells were seeded at 60% confluence and transfected with pCMV-VSVg and R7dEnvGFP using polyethylenimine (PEI). 48h later, the supernatant containing virus particles was harvested, filtered through a 0.45-um filter (Milipore) and added to cells with or without 8ug/ml dextran. Cells were infected by 2hr spinoculation at 1200rpm, 13°C, followed by 1hr incubation at 37°C. After incubation, cells were spun down, resuspended in media with or without dex and ethanol.

Reactivation assays

J-Lat cells were reactivated by 24h treatment with 1 uM of TSA (Sigma-Aldrich), 10 ng/ml TNF α (Peprotech), or 10 nM TPA (Cell Signaling Technologies). Primary CD4⁺ T cells were reactivated by placing cells on pre-coated anti-CD3 and anti-CD28 wells in a 96 well U-bottom plate for 48 hours. Cells were cultured in the continued presence of IL-2.

Flow cytometry / FACS analysis

To measure infectivity, 10^5 - 10^6 cells were isolated 48-72h after infection, fixed in 1% formaldehyde, and analyzed using a BD FACSCanto II flow cytometer. Live cell populations were defined by forward scatter versus side scatter plots and percent infectivity was measured by GFP expression. To determine the activation status of CD4⁺ T cells before and after 3 days of stimulation on anti-CD3 and anti-CD28 antibodies, 10^5 cells were stained with anti-human CD69-PE (eBioscience) and resuspended in PBS. All analyses were performed using FlowJo software. For FACS analysis, cells were

resuspended in PBS and GFP-positive cells were sorted using a FACS Aria Sorter. Collected cells were spun down and resuspended in media with 40% FBS.

Quantitative real-time PCR

For real-time PCR studies, samples from dex, ethanol, or untreated cells were collected at various time points throughout the experiment. Cells were treated with RNase A and genomic DNA was extracted according to the DNeasy Blood & Tissue Kit protocol (Qiagen) and the concentration was determined using a NanoDrop 1000 instrument (Thermo Scientific). 200ng of genomic DNA from each sample was digested with DpnI (New England BioLabs) for 4-5 hours. Real-time PCR was performed using previously published primers for proviral DNA and B-actin (Butler et al., 2001, Wu et al., 2006). Primers for GFP were also used.

CHAPTER FOUR

REACTIVATION OF LATENTLY INFECTED CELLS

INTRODUCTION

One current method for eliminating the latent reservoir is this “shock and kill” approach, where the virus is purged out of latency by drugs that reactivate latently infected cells. If this method is going to be used therapeutically, potential inhibitors of viral reactivation need to be considered. As stress and alcohol are common among HIV infected patients, it will be important to determine if these two stimuli affect the reactivation of latently infected cells.

The specific effects of these stimuli are complex and depend on a number of factors, such as treatment dose and duration. Many studies suggest that chronic and acute glucocorticoid treatments and alcohol exposure may elicit different effects. It is possible that different concentrations of dexamethasone and ethanol may have varying effects on proviral reactivation. We hypothesized that viral reactivation by latency reversing agents would be altered by ethanol and dexamethasone treatments.

EXPERIMENTAL DESIGN

J-Lat cell model

To test our hypothesis, we used the J-Lat cell model. These cells harbor transcriptionally silent HIV provirus that encodes GFP in place of *nef*. J-Lat cells were treated with dexamethasone or ethanol for at least 24 hours, then reactivated using a number of previously tested latency reversing agents (**Fig. 1, Table 1**). Reactivation was determined by flow cytometric analysis of GFP expression. Flow data were analyzed using FlowJo software.

Primary cell model

CD4⁺ T cells were isolated from PBMCs using lymphocyte separation media, activated with plate-bound anti-CD3 (2.5 ug/mL) and anti-CD28 (2.5 ug/mL) for 3 days, then infected by spinoculation with a VSVg pseudotyped HIV-1 reporter virus. 1 hour after spinoculation, cells were spun down and resuspended in new media +/- dex (10⁻⁶ M) or ethanol (17 mM). 72 hours post-infection, an aliquot of cells from each treatment group (NT, dex, ethanol) were fixed and GFP expression was analyzed by flow cytometry. 7 days after infection, cells were reactivated with anti-CD3 (2.5 ug/mL) and anti-CD28 (2.5 ug/mL). As a negative control, parallel cultures were plated in the absence of anti-CD3 and anti-CD28. 48h later, GFP expression and proviral DNA content were analyzed for each treatment group.

Drug	Mechanism of action	Concentration
Dexamethasone	Synthetic glucocorticoid	10^{-5} M – 10^{-10} M
Ethanol		17 mM – 65 mM
TSA	HDAC inhibitor	1 μ M
TNF α	NF- κ B activator	10 ng/ml
TPA	PKC activator	10 nM

Table 1. Drugs used for reactivation experiments.

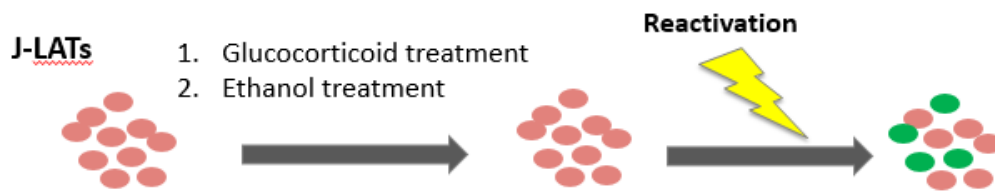


Figure 1. Schematic of J-Lat experimental design

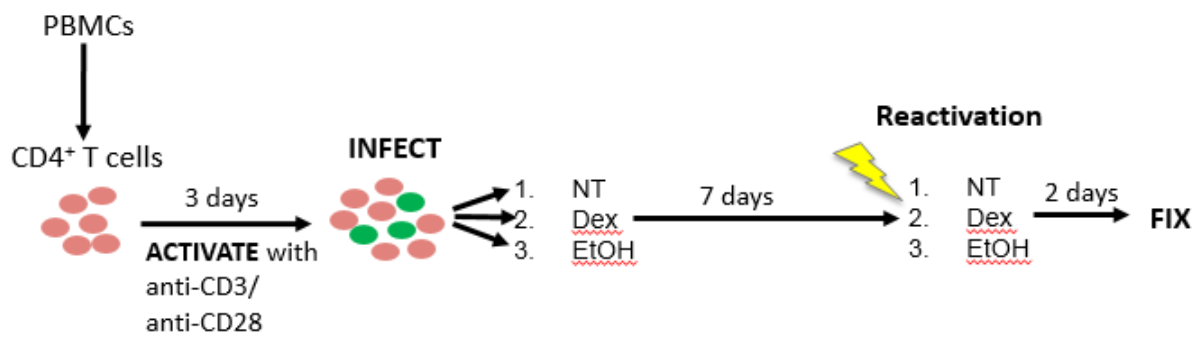


Figure 2. Schematic of primary cell experimental design

RESULTS

To determine how stress and alcohol affect viral reactivation, we used the J-Lat model. This is an *in vitro* cell model system to study mechanisms of HIV latency and the reactivation of latent proviruses. These cells were established by infecting Jurkat T cells with a GFP reporter virus and sorting all the GFP-negative cells, which essentially contain uninfected and latently infected populations (Kauder et al., 2009; Spina et al., 2013). Cells were then treated with TNF α and the GFP-positive cells were sorted and cloned (Kauder et al., 2009). Under basal conditions, J-Lats express little or no GFP, so the only way to determine what effects dexamethasone and ethanol treatments have on these cells is by reactivating them. J-Lat cells reactivate in response to various stimuli (**Fig. 3**). Preliminary results suggested that both dexamethasone and ethanol treatments result in slight decreases in J-Lat reactivation, particularly with TSA and TPA (**Fig. 4**).

We next examined if this inhibition by ethanol was dose dependent. Physiologically relevant ethanol concentrations were tested. With increasing ethanol concentrations, there was a slight dose dependent decrease in J-Lat reactivation by TPA (**Fig. 5**). This result was consistent across multiple experiments (data not shown). A very subtle decrease in J-Lat reactivation was also seen with TSA. However reactivation with TNF α never yielded a dose dependent decrease. Typically, the number of cells reactivated with TNF α was consistent across all ethanol concentrations tested but sometimes a slight increase in reactivation was seen.

To determine if these results changed over time, we cultured J-Lats with low and high concentrations of dex and ethanol for two weeks. Every day, cells were supplemented with media +/- dex or ethanol and then reactivated with TSA, TNF α , or

TPA. Dex and ethanol treatments consistently inhibited reactivation by TPA, while reactivation with TSA and TNFa closely mimicked the control (**Fig. 6**).

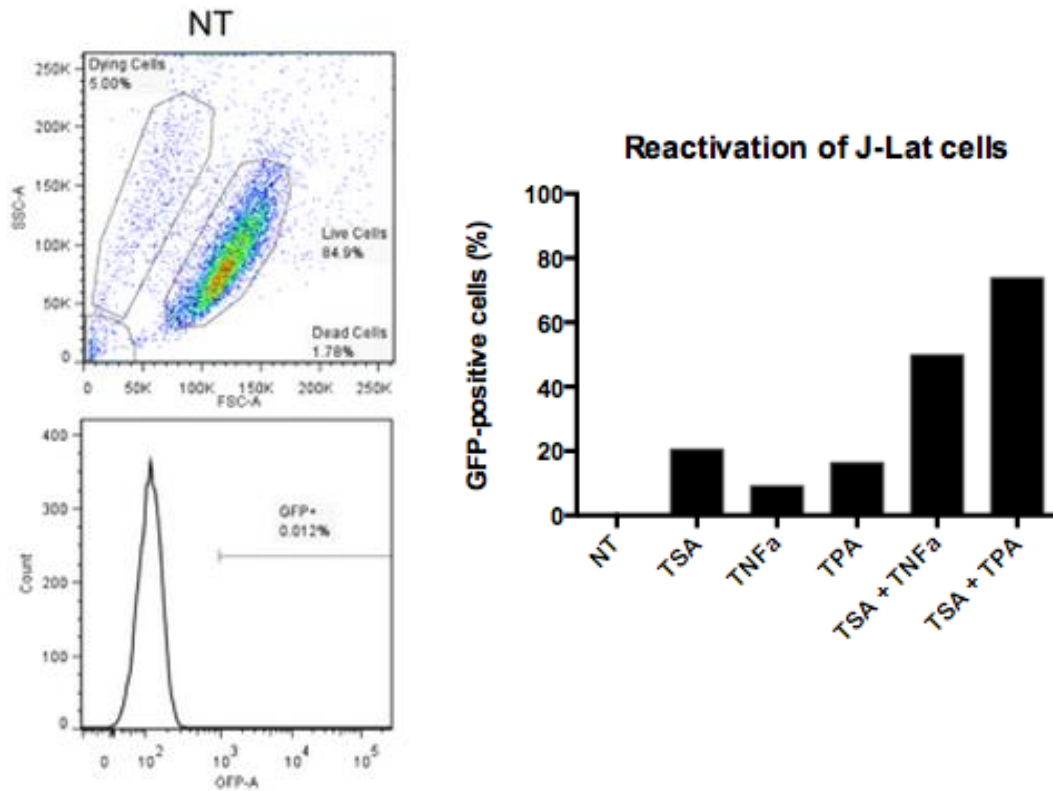


Figure 3. J-Lat reactivation by latency reversing agents. J-Lat cells were left untreated or were treated with TSA (1 μ M), TNF α (10ng/mL), TPA (10nM) and combinations of these drugs for 24 hours. GFP expression was analyzed by flow cytometry to determine the percent of cells reactivated by the indicated drug(s).

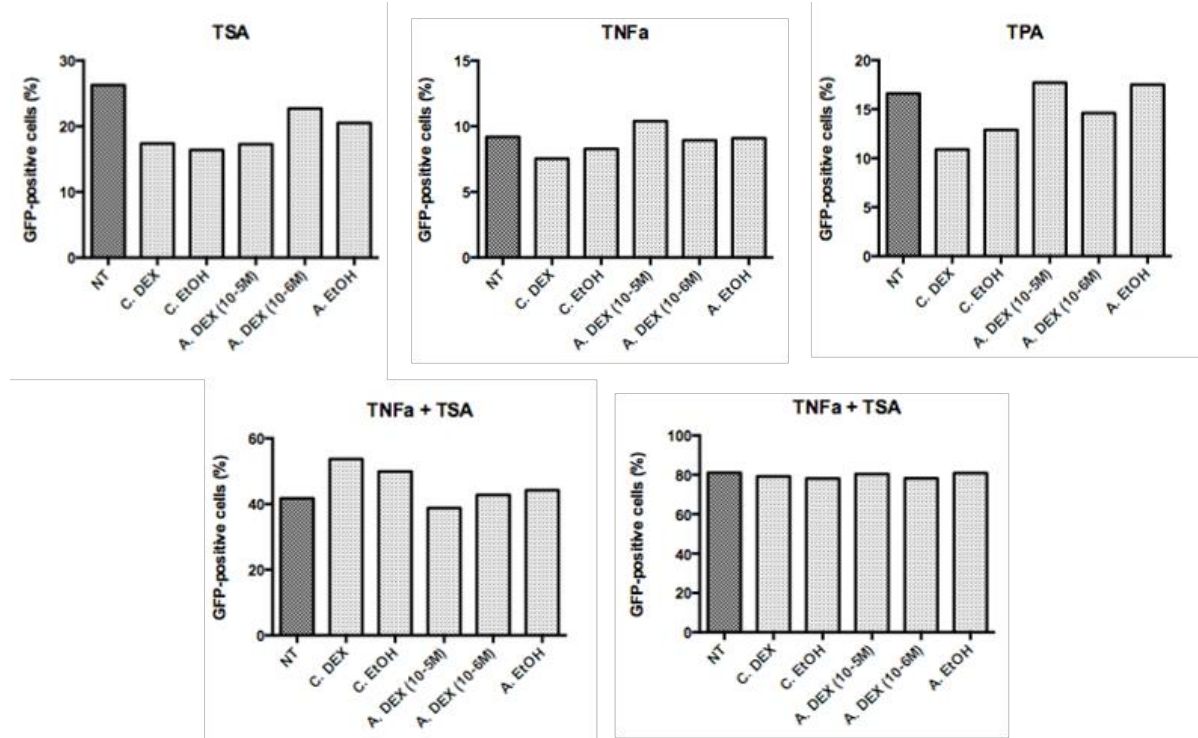


Figure 4. J-Lat reactivation in response to dexamethasone and ethanol exposure. J-Lat cells were pre-treated with acute dexamethasone (10^{-5} M or 10^{-6} M, 24h) acute ethanol (170mM, 24h), chronic dexamethasone (10^{-10} M, 4 days) or chronic ethanol (170mM, 4 days). Following dex / EtOH treatments, cells were reactivated with TSA, TNF α , TPA or combinations of these drugs. Each graph depicts flow cytometry results from reactivation by a particular drug or combinations of drugs. The percent of cells reactivated (y-axis) in response to different dex / EtOH treatments (x-axis, light grey bars) was compared to the no-treatment control (x-axis, dark grey bar).

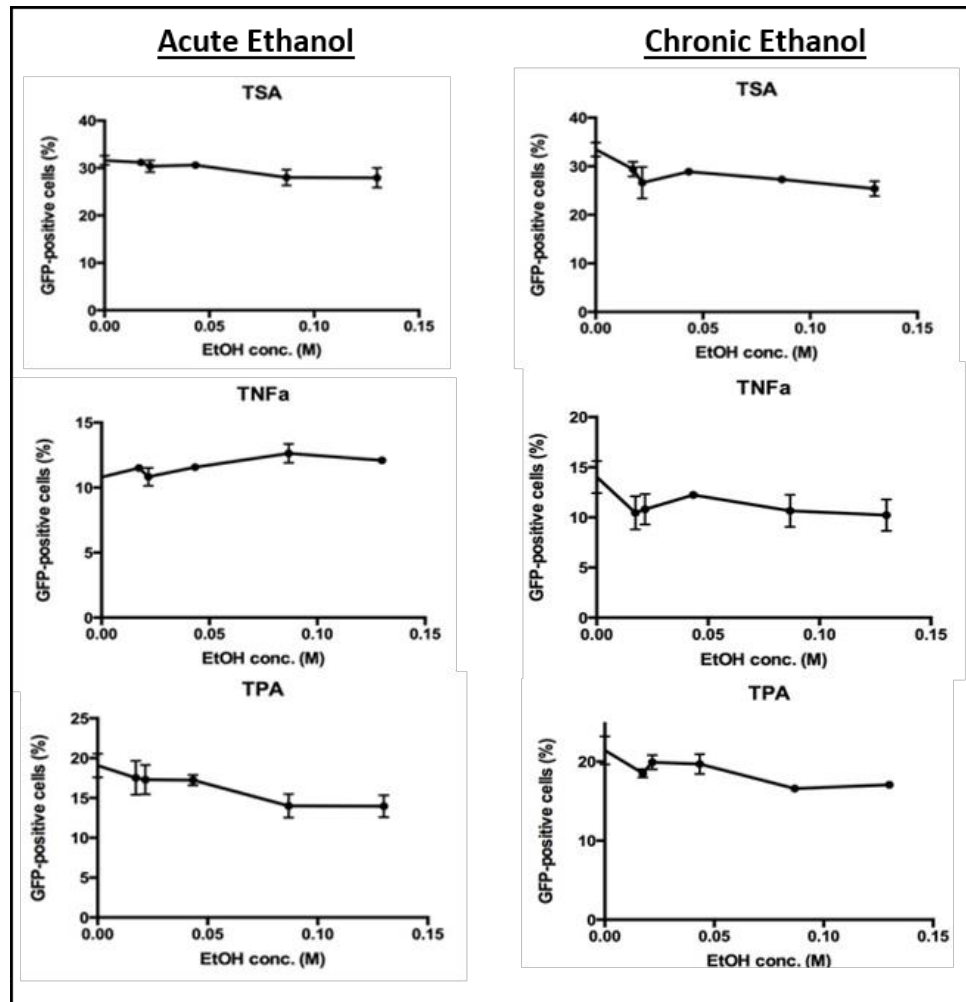


Figure 5. J-Lat reactivation in response to increasing ethanol concentrations. J-Lats were treated with ethanol (0.0173 M, 0.0217 M, 0.0434 M, 0.0686 M, 0.13 M) for 24 hours (acute treatment) or 4 days (chronic treatment). Following ethanol treatments, cells were given an additional dose of ethanol, then reactivated with TSA, TNFa, or TPA in triplicates. Proviral reactivation was measured by flow cytometry. Data are represented as mean \pm SEM.

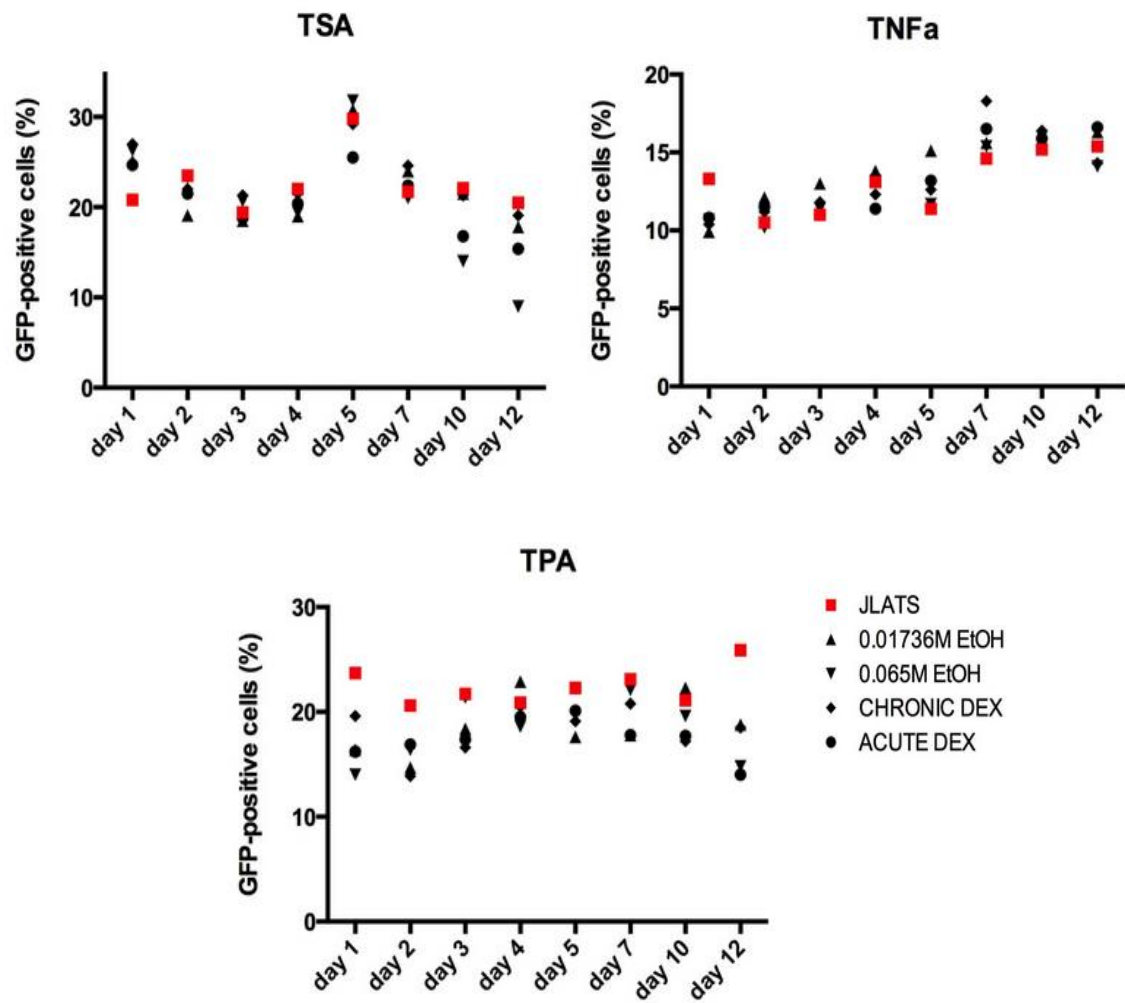


Figure 6. Long-term dex and ethanol exposure reveals modest decreases in J-Lat reactivation by TPA. J-Lats were treated with acute dex (10^{-5} M), chronic dex (10^{-10} M) and ethanol (0.01736 M and 0.065 M) every 24 hours for 2 weeks. Aliquots of cells were reactivated with TSA, TNFa, and TPA every 1-3 days, and GFP expression was analyzed. Graphs depict viral reactivation (y-axis) by a specific LRA, in response to dex / EtOH exposure at the indicated time points (x-axis).

We next repeated these experiments using a primary cell model. In short, purified CD4⁺ T cells were isolated from PBMCs and stimulated with α -CD3/CD28, mimicking T cell activation *in vivo*. Activation was measured by CD69 expression. CD69 is a cell surface marker rapidly upregulated after T cell activation, making it an ideal marker for determining the activation status of these T cells after three days of stimulation (**Fig. 7**). Following 3 days of activation, cells were infected and treated with or without dex (10^{-6} M) or ethanol (17 mM) for 7 days. Aliquots were taken for flow and real-time PCR analysis at various time points throughout the experiment.

There is a marked reduction in GFP expression in all treatment groups between 3 and 7 days post-infection (**Fig. 8**). This loss of GFP expression is indicative of proviruses becoming latent. If these viruses are latent, there should be potent reactivation in response to anti-CD3 and anti-CD28 stimulation. However we did not see a change in GFP expression in reactivated cells compared to unactivated cells (**Fig. 9**).

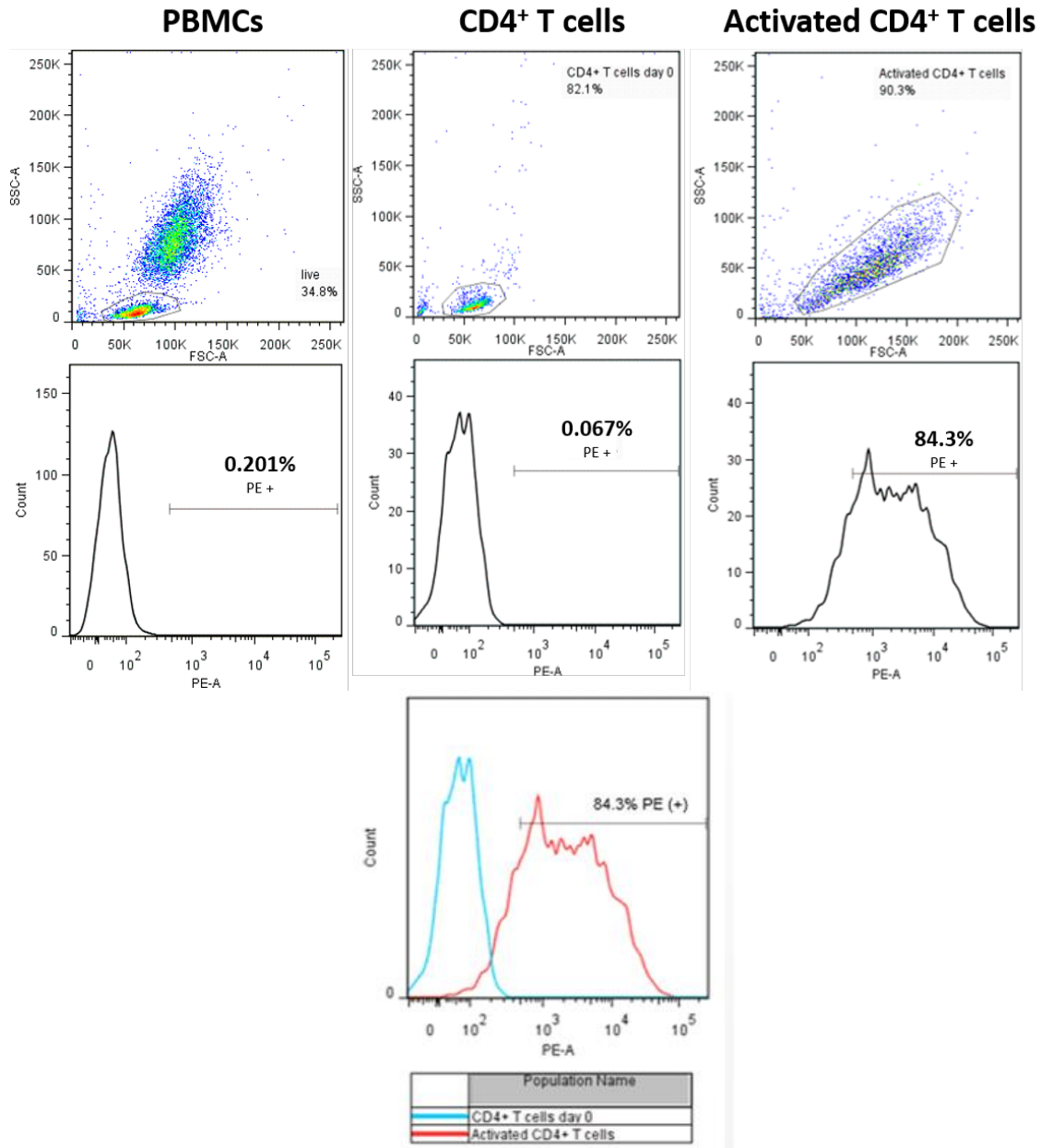


Figure 7. Isolation and activation of CD4⁺ T cells. Representative dot plots and histograms of PBMCs prior to CD4⁺ T cell isolation (top left) and after isolation of CD4⁺ T cells (top middle). CD4⁺ T cell activation was measured by CD69 expression (right). Merged histograms show increased CD69 expression prior to and after T cell activation (bottom).

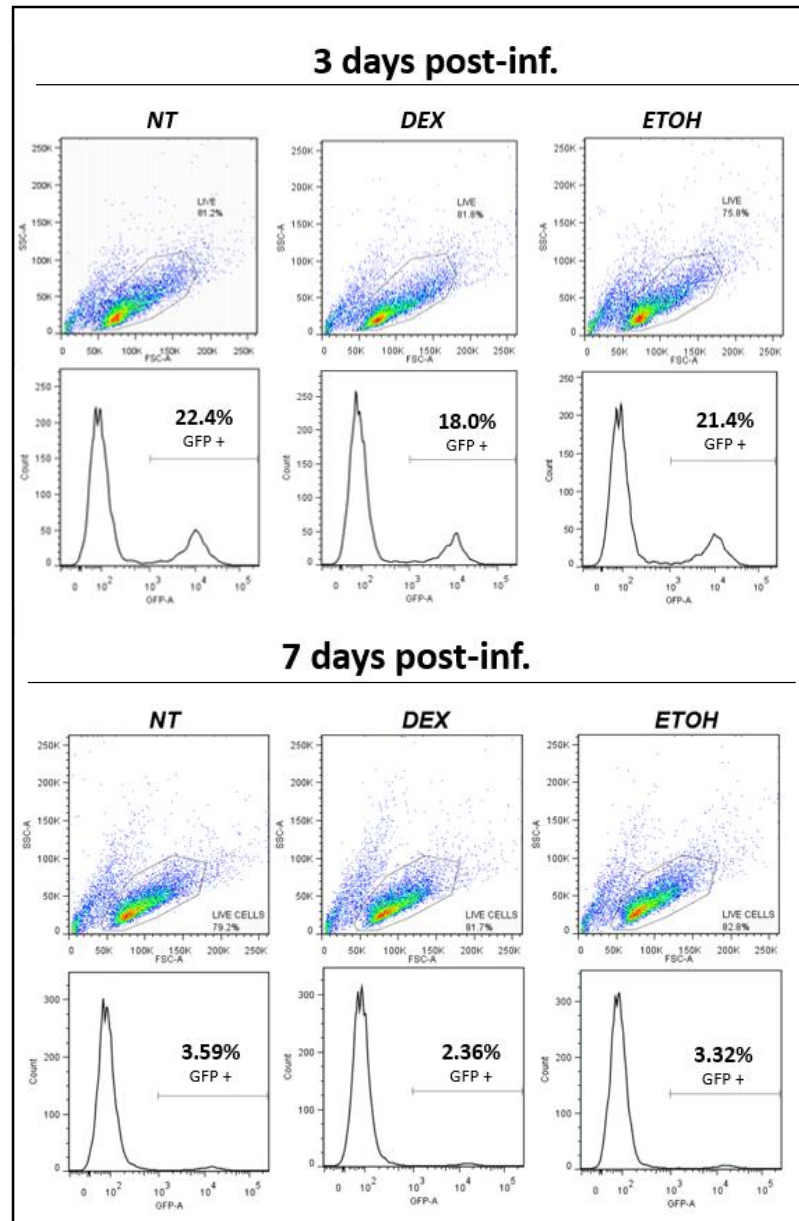


Figure 8. CD4⁺ T cells lose their GFP expression 1 week post-infection. 1hr after infection, cells were resuspended in new media with or without dex (10^{-6} M) or EtOH (17 mM). Aliquots of cells from each treatment group were fixed 3 and 7 days after infection and GFP expression was determined. Dot plots and histograms show the percent of GFP-positive cells in each treatment group at the indicated time points after infection.

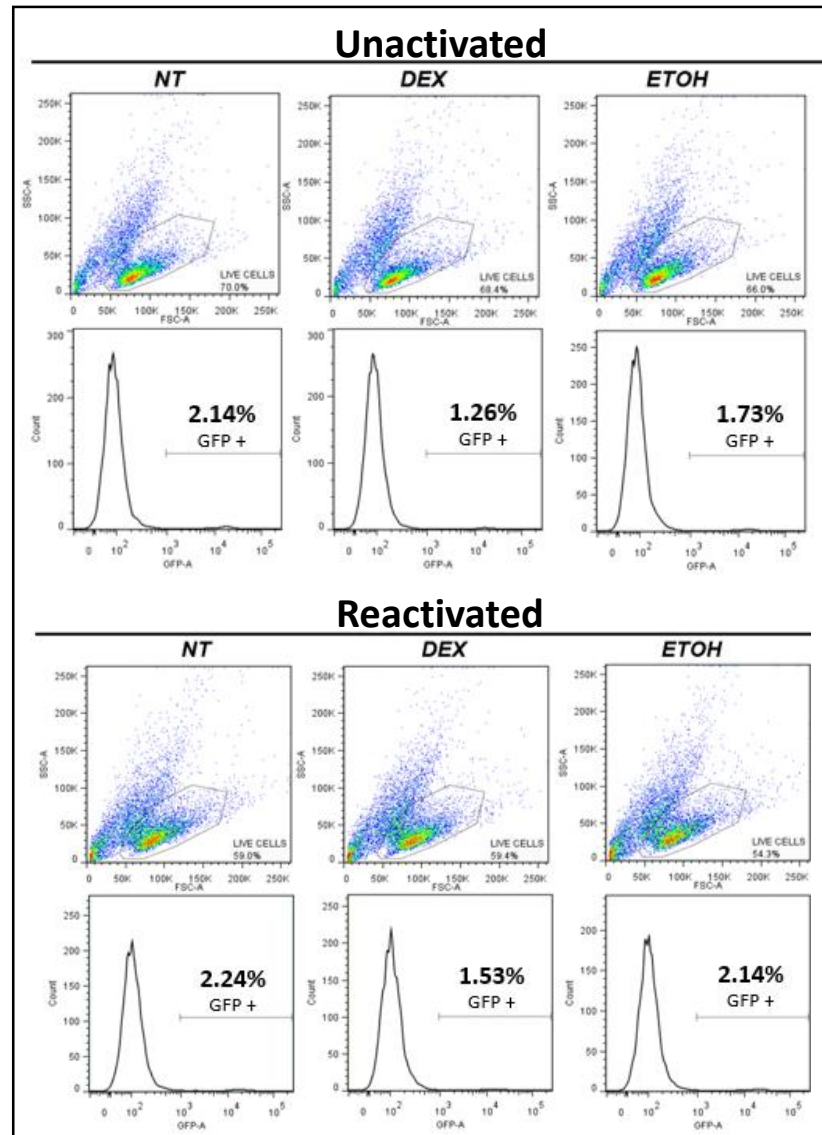


Figure 9. No significant change in GFP expression between reactivated and unactivated cells, regardless of treatment conditions. Cells were treated with dex (10^{-6} M), EtOH (17 mM) or left untreated for 7 days. Cells from each treatment group were either activated with anti-CD3 and anti-CD28 or left unactivated. Cells were fixed 48 hours later. Dot plots and histograms show the percent of GFP-positive cells in each treatment group.

DISCUSSION

The fundamental issue with this J-Lat experiment is that it depends on the reactivation of latent cells by potent drugs in order to see any kind of effect dexamethasone or ethanol treatments may be having. This is problematic for two reasons. First, many of these drugs are toxic to J-Lats, making data analysis difficult since only a small percentage of cells will survive treatments with dexamethasone or ethanol plus reactivating drugs. Lowering drug dosage or duration is not a solution, because cells do not reactivate at lower concentrations or shorter durations. Second, even if dexamethasone and ethanol are affecting latency as we predict, we may be unable to see such effects using this experimental design. Our preliminary studies using J-Lats were mostly inconclusive, so it was important to test our hypothesis using a primary cell model. Evidence for this is supported by the previous finding that glucocorticoids suppress the induction of HIV replication in latently infected primary CD4⁺ T cells (Chun et al., 1998).

Unfortunately, our results with primary cells were also inconclusive. Infected cells lost their GFP expression over time but were unable to be reactivated. Since the premise of this experiment relies on our ability to reactivate these cells, it is impossible to determine if these drug treatments affect viral reactivation.

CHAPTER FIVE

ESTABLISHMENT OF LATENCY

INTRODUCTION

Alcohol exposure and stress are commonly associated with HIV infected individuals, and both stimuli are likely to be involved during the early stages of HIV infection (i.e. alcohol consumption correlated to risky behavior and HIV contraction or stress associated with testing HIV-positive) (Justice et al., 2013). As discussed above, latency is established in CD4⁺ T cells as they become quiescent, forming long-lived memory T cells. The silencing of viral expression is mediated by a number of mechanisms including the absence of host transcription factors and epigenetic modifications. Since stress and alcohol exposure alter transcription factor activity and the chromatin environment, it is possible that dexamethasone and ethanol affect the formation of the latent reservoir. We hypothesized that the number of cells driven into latency would be altered when cells are treated with dexamethasone or ethanol.

EXPERIMENTAL DESIGN

Jurkat cells

Jurkat T cells were infected with VSVg pseudotyped HIV-1 reporter virus and the GFP positive cells were sorted. Treatments were started 24 hours after FACS and lasted 50 days. Every day, cells were given dex (10⁻⁵ M), ethanol (65 mM) or left untreated.

Every 3-4 days, cells were fixed and GFP expression was analyzed by flow cytometry to monitor the loss of GFP expression over time. At various time points throughout the experiment, aliquots of cells were taken for real-time PCR experiments. Genomic DNA was extracted and digested with DpnI. Real-time PCR was performed for each sample using LateRT, GFP, and B-actin primers.

Primary cell model

CD4⁺ T cells were isolated from PBMCs using lymphocyte separation media, activated with plate-bound anti-CD3 (2.5 ug/mL) and anti-CD28 (2.5 ug/mL), and infected with a VSVg pseudotyped HIV-1 reporter virus. To enhance infectivity, DEAE-Dextran was added to virus cultures at a final concentration of 8 ug/mL. 72 hours after infection, GFP-positive cells were sorted. Sorted cells were cultured together for an additional 24 hours in serum-rich media with 20 U IL-2. Cells were then divided into three groups and treated with dex (10^{-6} M), ethanol (65 mM), or left untreated. 1 week later, cells were resuspended in PBS and GFP expression was analyzed by flow cytometry. Genomic DNA was extracted from remaining cells and digested with DpnI. Real-time PCR was performed for each sample using LateRT, GFP, and B-actin primers.



Figure 10. Schematic of Jurkat experimental design

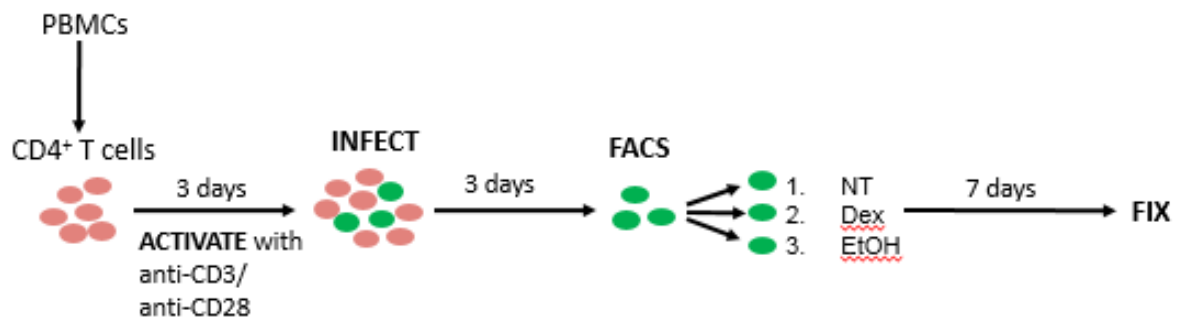


Figure 11. Schematic of primary cell experimental design

RESULTS

Preliminary data suggested that both dexamethasone and ethanol enhance the size of the latent reservoir. There was a substantial increase in the percent of GFP-negative cells when cells were treated for 6 weeks with dexamethasone or ethanol compared to those left untreated (**Fig. 12**).

It is possible that the decrease in GFP expression is due to the presence of uninfected cells that are out-competing the GFP-positive, infected cells. If this is the case, there will be no provirus present in these GFP-negative cells. However, latently infected cells will still contain integrated provirus. We did real-time PCR to confirm that the loss of GFP expression in dex and ethanol treated cells was due to proviral silencing. There was an evident decrease in the proviral copy number in dex and ethanol treated cells compared to cells left untreated (**Fig. 13**). The decrease in integrated provirus correlates with the loss of GFP expression seen in **Fig. 12**. These data suggest that the latent reservoir is not increased by dex or ethanol treatments. Real-time PCR was repeated using primers to genes downstream of the viral promoter to ensure that epigenetic modifications were not interfering with primer annealing or SYBR green binding. Similar results were seen (data not shown).

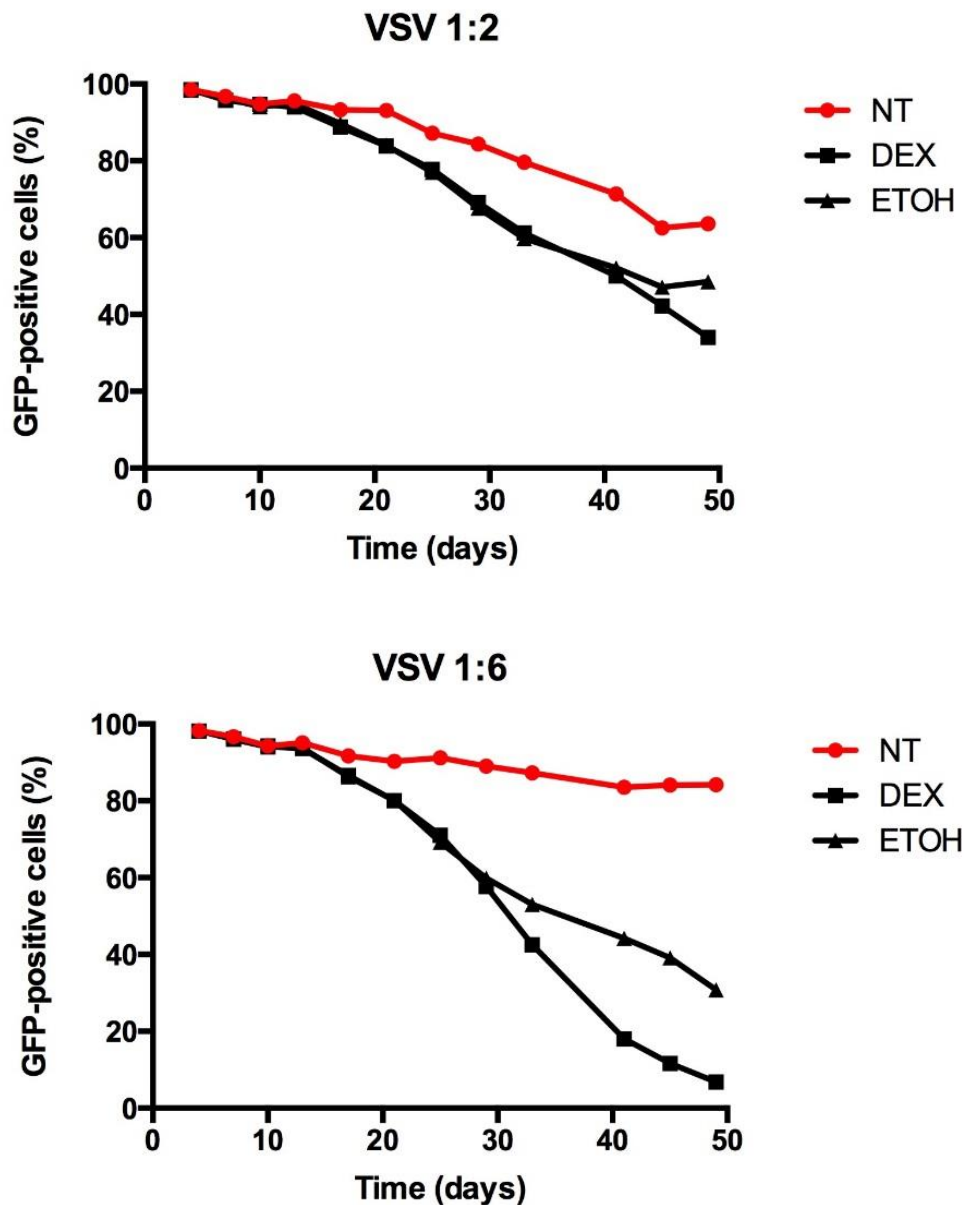


Figure 12. Infected Jurkat cells lose GFP expression over time. GFP-positive sorted cells were treated with dex (10^{-6} M) or ethanol (65 mM) for 6 weeks. Graphs depict the changes in GFP expression in cells treated with dex / EtOH (black lines) compared to untreated cells (red lines).

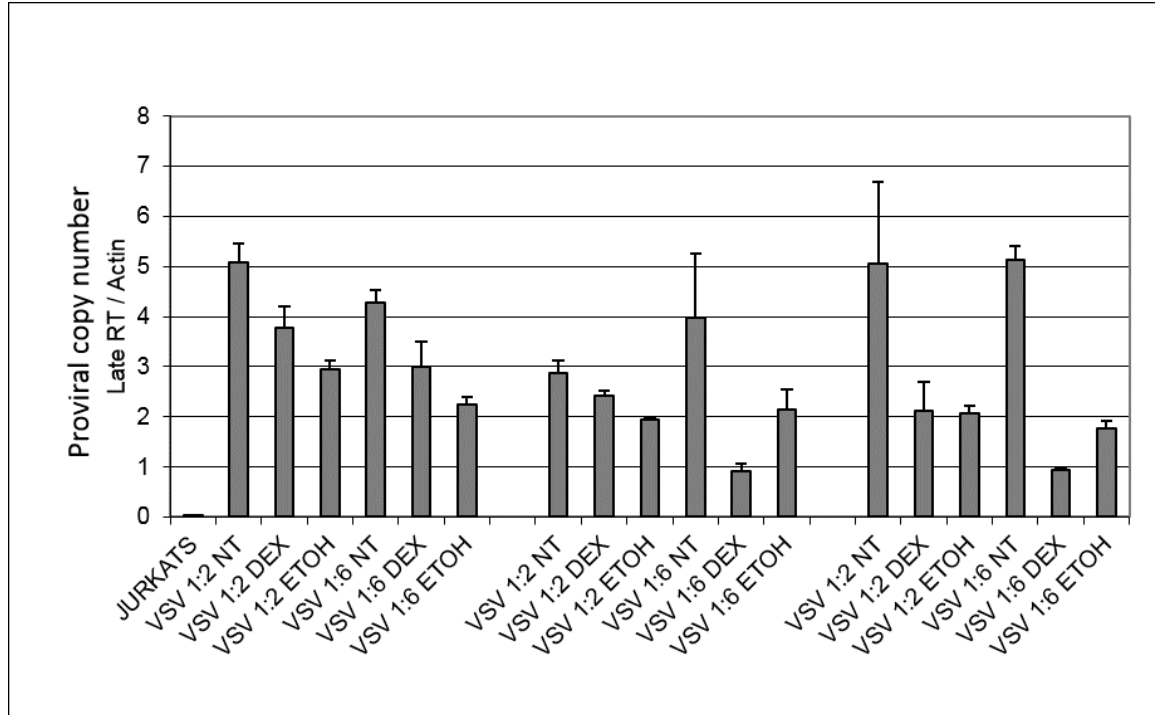


Figure 13. Loss of proviral DNA in dex and ethanol treated cells. GFP-positive sorted cells were treated with dex (10^{-6} M) or ethanol (65 mM). 30, 35, and 40 days after sorting, pellets were collected from dex, EtOH, and untreated cells. Genomic DNA was extracted and digested with DpnI. Real-time PCR was performed for each set of samples in triplicate using primers for proviral DNA and B-Actin. Graph depicts viral copy number normalized to actin.

This experiment was repeated using latently infected primary CD4⁺ T cells. Briefly, CD4⁺ T cells were isolated from PBMCs and activated with anti-CD3 and anti-CD28. Once activated, cells were infected and GFP expression was analyzed 48h after infection (**Fig. 14**). GFP-positive and negative cells were sorted 72h post-infection. 24 hours after sorting, cells were treated with dex and ethanol. 1 week later, cells were analyzed for GFP expression and the amount of viral DNA present by flow cytometry and real-time PCR, respectively (**Fig. 15-16**). There was a modest reduction in GFP expression in the untreated group and an even more prominent reduction in the dex-treated group, as seen with the Jurkats. Ethanol-treated cells died during the experiment. Real-time PCR data revealed differences in the amount of proviral DNA present in dex vs. untreated samples. Surprisingly, there was more viral DNA in the dex-treated cells compared to the untreated group. As expected, there was no viral DNA in the GFP-negative sorted cells.

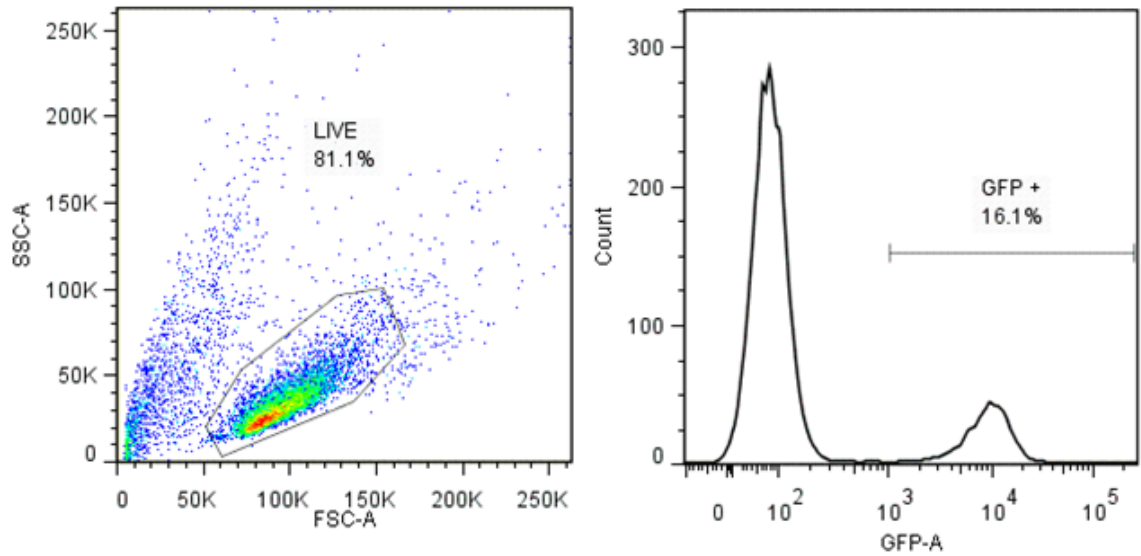


Figure 14. Primary cells infected with VSVg pseudotyped HIV-1 reporter virus.

48h after infection, cells were fixed in 1% formaldehyde and GFP expression was measured by flow cytometry. 16.1% cells were productively infected, as indicated by GFP expression.

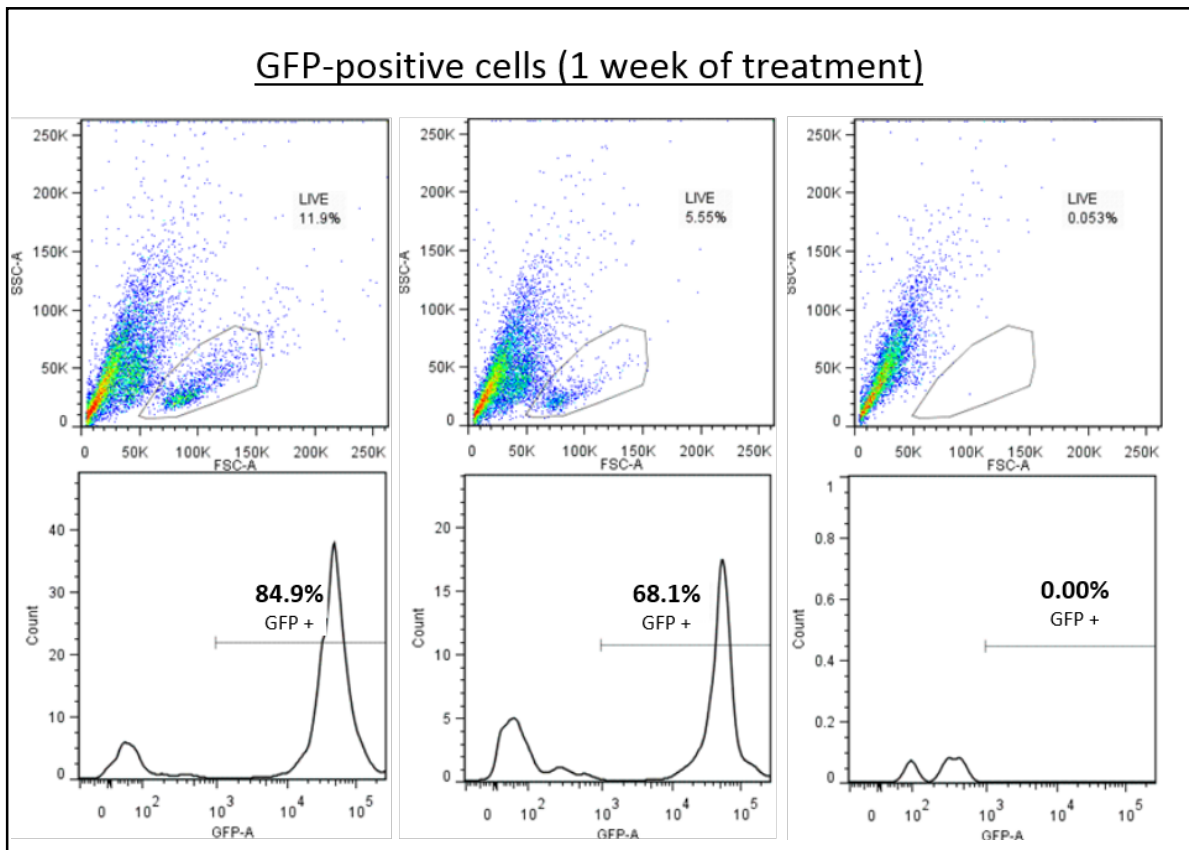


Figure 15. GFP expression in GFP-positive sorted cells one week after FACS. GFP-positive sorted cells were treated with dex (10^{-6} M), ethanol (65 mM), or left untreated for 1 week. Cells were resuspended in PBS and GFP expression was analyzed by flow cytometry. Dot plots and histograms show the percent of GFP-positive cells within the live cell population.

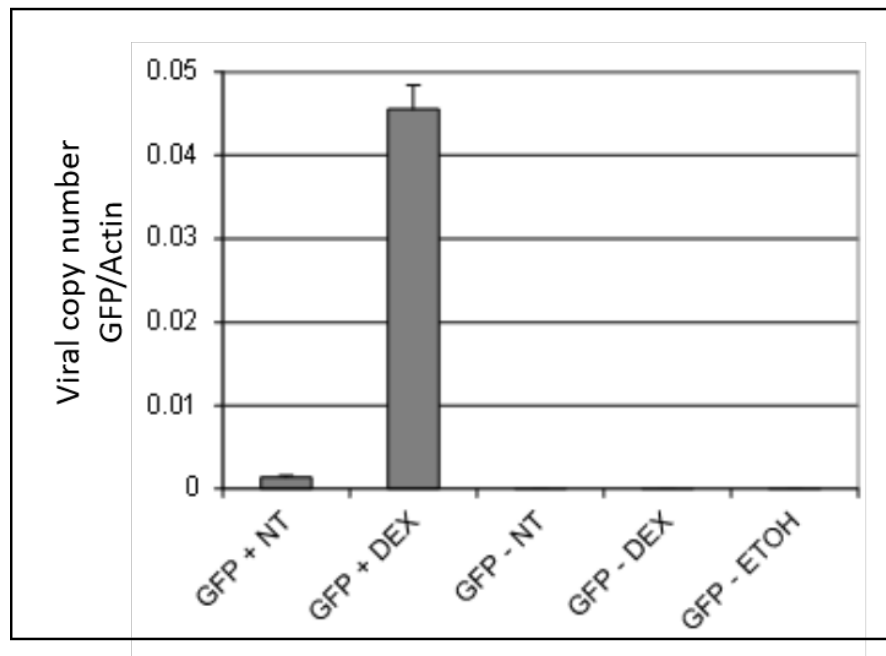


Figure 16. Increased proviral DNA in dex-treated cells compared to the untreated group. GFP-positive and negative sorted cells were treated with dex (10^{-6} M), ethanol (65 mM), or left untreated for 1 week. Genomic DNA was extracted and digested with DpnI. Real-time PCR was performed for each samples using GFP and B-actin primers. Graph depicts proviral copy number normalized to actin.

DISCUSSION

Real-time data from Jurkat experiments revealed that the loss of GFP expression seen in our flow data was not due to these viruses become latent, rather an increased population of GFP-positive cells in our cell culture. This is a surprising result, as all cells (NT, dex, and EtOH) came from the same population of productively infected, GFP-positive cells. It is possible that some GFP-negative cells were mistakenly sorted into this GFP-positive cell population, and these drug treatments provided some kind of selective advantage to the uninfected cells. It is also probable that dex and ethanol treatments are enhancing the death of the infected cell population.

In the primary cells, we saw similar flow data where dex-treated cells lose their GFP expression more quickly than untreated cells. However, real-time PCR data revealed increased proviral DNA content in cells treated with dexamethasone. The proviral copy number in this RT-PCR data is abnormally low, particularly in the untreated cells, suggesting a problem with this data set. We were unable to determine the effects of ethanol, as these cells died during the experiment.

CHAPTER SIX

CONCLUSIONS AND FUTURE DIRECTIONS

In these studies, we attempted to determine the effects of stress and alcohol on HIV latency by examining how latency gets established and reactivated in the presence of glucocorticoids and ethanol. Our initial experiments with J-Lat cells showed modest reductions in viral reactivation in response to dexamethasone and ethanol exposure. We saw reproducible inhibition in viral reactivation with TPA after acute and chronic dexamethasone and ethanol treatments. Other drugs tested did not seem to have this effect. We were able to successfully establish a latent state in primary CD4⁺ T cells as indicated by a pronounced reduction in GFP expression 1 week after infection. However, data from these experiments were inconclusive since the cells were not reactivated, despite stimulation with α CD3 and α CD28.

We used a Jurkat cell model to examine how latency gets established in the context of dexamethasone and ethanol. These cells appeared to become latent within 50 days after infection, and it seemed that both dexamethasone and ethanol were enhancing the establishment of this latent state. Real-time PCR revealed a reduction in proviral copy number in these treated cells, compared to the no treatment control. Together, these data indicate that latency was not established in these cells, rather there was a loss of infected cells and/or an increase in uninfected cells in the dexamethasone and ethanol treated

cultures. We developed a model for examining this in primary cells, but a lot of cells were lost during the infection and sorting processes. We did see a slight reduction in GFP expression within a week after FACS, and the reduction in GFP expression was more pronounced in the dexamethasone-treated cells. Real-time data revealed increased viral DNA content in the dexamethasone-treated cells compared to the no treatment group. However, due to the very low proviral copy number in these cell populations, we are not sure if this result is real.

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VITA

Sarah Ilene Talley was born in New Orleans, LA. She completed her secondary education at the Academy of the Sacred Heart and continued her studies at Rhodes College where she earned a Bachelor of Science in Chemistry. After graduation, she was employed at St. Jude Children's Research Hospital and spent three months working in an infectious disease clinic in Tanzania.

In the fall of 2013, Sarah entered the Infectious Disease and Immunology MS program at Loyola University Chicago. She joined the laboratory of Dr. Edward Campbell where she began investigating potential cofactors of HIV latency. Sarah will be continuing her work at Loyola next year, in pursuit of her Ph.D.

